IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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In re the Application of: **KATO, et al**

Serial No.: **09/340,196** Group Art Unit: **1642**

Filed: **June 28, 1999** Examiner:**Jennifer E.Hunt**

FOR: **METHOD FOR MEASURING THYROGLOBULIN**

considered 5/5/2003 AH

DECLARATION

Commissioner of Patents and Trademarks
Washington, DC 20231

Sir:

I, Kenji NAKAMURA, a Japanese citizen, residing at Suzukakedai, Sanda-shi, Hyogo, Japan do hereby solemnly and sincerely declare and state:

THAT I am by profession a research chemist having been awarded a master's degree from the graduate course of the Pharmacology, the Department of Pharmacology, Nagasaki University, in March, 1984;

THAT I began employment with Wako Pure Chemical Industries, Ltd., the Assignee of the above-identified application, in April, 1984 and have been engaged, since that time, in research and study of immunoassay, especially on the development of diagnostics thereof in the Osaka Research Laboratories;

THAT I am a co-inventor of the invention disclosed in the above-identified U.S. patent application and am well aware of the prosecution history thereof;

THAT, I conducted the following Experiment with my best knowledge honestly and sincerely.

Experiment

※Hereinafter, thyroglobulin is abbreviated as Tg.

1. Materials

[Sample]

The human serums were collected 9 from normal patients, 12 from benign thyroid adenoma patients and 29 from thyroid carcinoma (malignant) patients, and they were used as the samples.

[Reagent l]

A 50 mM MOPS (3-(N-morpholino) propanesulfonic acid) buffer solution (Good's Buffer, pH 7.5, containing 0.15 M NaCl) containing 10% Bloc Ace (decomposition product of casein, trade name, manufactured and sold by Dainippon Pharmaceutical Co. Ltd.) and 1% mouse serum (manufactured and sold by Dainippon Pharmaceutical Co. Ltd.) previously inactivated at 56℃ for 30 minutes was prepared, which was used as Reagent l.

[Reagent 2]

A 200 mM of boric acid buffer solution (pH 8.5, containing 2mM EDTA-3Na) containing 5mM luminol (manufactured and sold by Wako Pure Chemical Industries, Ltd.) was prepared, which was used as Reagent 2.

[Reagent 3]

A 1 mM of phosphate buffer solution (pH 3 containing 2mM EDTA-2Na) containing 0.02% hydrogen peroxide was prepared, which was used as Reagent 3.

[anti Tg-1·Fab'-POD]

An anti Tg antibody (manufactured and sold by Dako Cytomation .Ltd., Clone No.DAK-Tg6), which do not bind to Tg bound to LCA (hereinafter abbreviated as "anti Tg-1"), was treated after a conventional manner to give Fab' fragments and then they were bound to peroxidase (manufactured and sold by Toyobo Co., Ltd.) after a conventional manner to give peroxidase labeled anti Tg-1 antibody Fab' fragments (hereinafter abbreviated as "anti Tg-1·Fab'-POD").

[Immuno bead]

An anti Tg antibody having different recognizable epitopes from anti Tg-1

(manufactured and sold by Dako Cytomation .Ltd., Clone No.TF-25, hereinafter abbreviated as "anti Tg-2") was treated after a conventional manner with a polystyrene bead to give a polystyrene bead on the surface of which anti Tg-2 antibody was immobilized was prepared (hereinafter abbreviated as "Immuno bead").

[Washing buffer]

5 mM phosphate buffer pH6.6 was used as a washing buffer of Immuno bead.

2. Measurement method

Samples in an amount of 50μL were mixed with 100μL of Reagent l and one of Immuno bead in each tubes, followed by allowing a reaction to take place at 8℃ for 40 minutes. After the reaction, the supernatant was removed, and each Immuno beads in each tubes were washed three times with the washing buffer, and 150μL of Reagent 1 containing 1mg/mL of LCA was added to each tubes, followed by allowing a further reaction to take place at 8℃ for overnight. Each Immuno beads in each tubes were washed three times with the washing buffer. 150μL of Reagent 1 containing 2×10^{-8} M of anti Tg-1・POD was added to the each tubes, followed by allowing a reaction to take place at 8℃ for 40 minutes to form a sandwich complexes of a "LCA"-"Tg bound to LCA"-"Immuno bead" complex or a "anti Tg-1・Fab'-POD"-"Tg not bound to LCA"-"Immuno bead" complex. After that, the supernatant was removed, and each Immuno beads in each tubes were washed for three times with the washing buffer. Reagent 3 in an amount of 100μL and 100μL of Reagent 4 was added to each tubes, luminescence of each of tubes were measured by Auto Lumat LB953 (Berthold Company). The measured luminescences were applied to a calibration curve showing a relationship between Tg amount and luminescence which was previously prepared by using a Tg solution containing a known amount of Tg after a similar manner, and an amount of Tg not bound to LCA was obtained.

The same measurements for the total Tg amount were conducted on the same samples after a same manner mentioned above except for using 150μL of Reagent 1 only in place of Reagent 1 containing LCA.

The amount of Tg bound to LCA was obtained by subtracting the amount of Tg not bound to LCA from the amount of total Tg.

Those results were applied to the following equation to calculate a ratio(%) of Tg

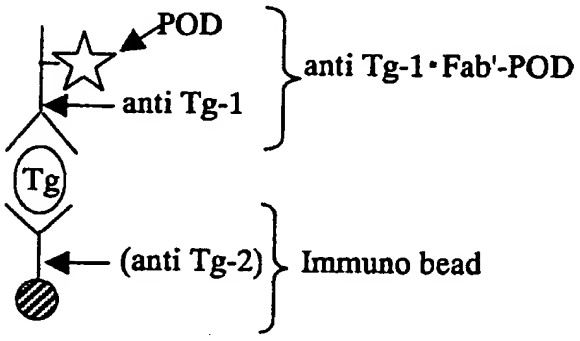
bound to LCA.

[Calculation of a ratio of Tg bound to LCA]

Ratio (%) of Tg bound to LCA=[(an amount of Tg bound to LCA) / (total Tg)]×100

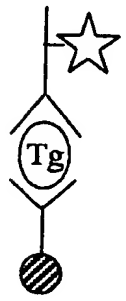
3. A principle of measurement in the present experiment

<measurement of Total Tg>

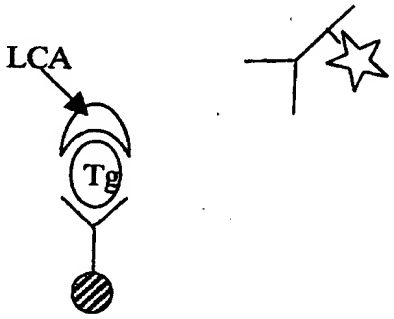


All of anti Tg-1・Fab'-POD can bind to all of Tg
when LCA is not in the reaction reagent

<measurement of Tg not bound to LCA>



Anti Tg-1 can bind to Tg
which LCA is not bound



Anti Tg-1 cannot bind to Tg
when LCA already bound to the Tg

4. Result

[1] Thus obtained an amount (ng/mL) of total Tg, an amount (ng/mL) of Tg bound to LCA, an amount (ng/mL) of Tg not bound to LCA and a ratio (%) of Tg bound to LCA / total Tg are shown in Figure 1 to 4, respectively. In Fig. 1 to 4, 1 of the axis of abscissa refers the case using the sample obtained from normal patients, 2 refers the case using the sample obtained from benign thyroid adenoma patients, and 3 refers the case using the sample obtained from thyroid carcinoma patients (malignant).

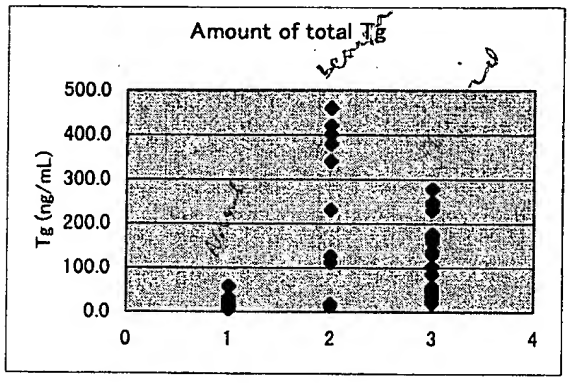


Fig. 1

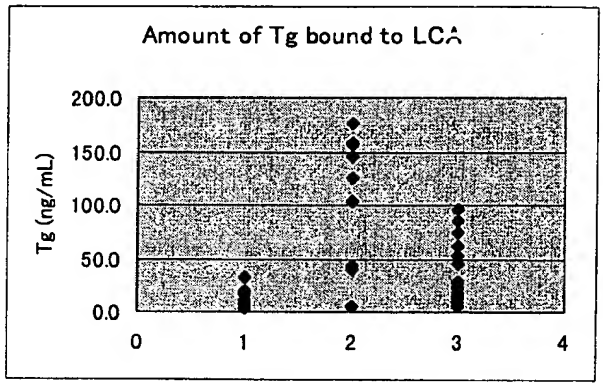


Fig. 2

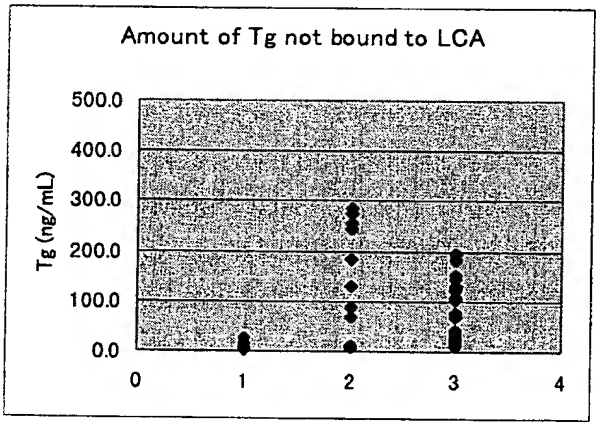


Fig. 3

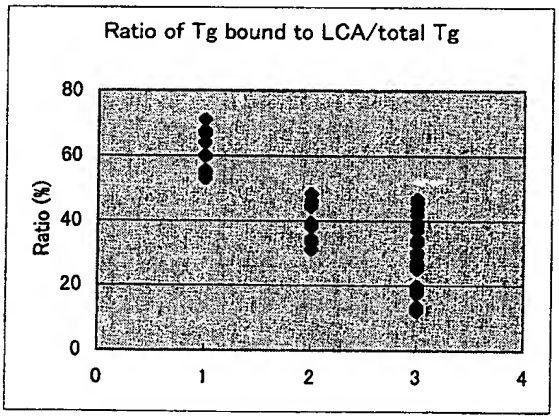


Fig. 4

As is clear from Fig. 1, the range of the amounts of total Tg in the serum of normal patients (1) is overlapped with that of benign thyroid adenoma patients (2) and that of thyroid carcinoma patients (3). The range of the amounts of total Tg in the serum of thyroid carcinoma overlapped with that of benign thyroid adenoma patients. From these results, it is

understood that differentiation diagnosis of thyroid carcinoma or benign thyroid adenoma from normal or that of thyroid carcinoma from benign thyroid adenoma or normal by using the amounts of total Tg is impossible.

As is clear from Fig. 2, the range of the amounts of Tg bound to LCA in the serum of normal patients (1) is overlapped with that of benign thyroid adenoma patients (2) and that of thyroid carcinoma patients (3). The range of the amounts of Tg bound to LCA in the serum of thyroid carcinoma overlapped with that of benign thyroid adenoma patients . From these results, it is understood that differentiation diagnosis of thyroid carcinoma or benign thyroid adenoma from normal or that of thyroid carcinoma from benign thyroid adenoma or normal by using the amount of Tg bound to LCA is impossible.

As is clear from Fig. 3, the range of the amounts of Tg not bound to LCA in the serum of normal patients (1) is overlapped with that of benign thyroid adenoma patients (2) and that of thyroid carcinoma patients (3). The range of the amounts of Tg not bound to LCA in the serum of thyroid carcinoma overlapped with that of benign thyroid adenoma patients. From these results, it is understood that differentiation diagnosis of thyroid carcinoma or benign thyroid adenoma from normal or that of thyroid carcinoma from benign thyroid adenoma or normal by using the amounts of Tg not bound to LCA is impossible.

Fig. 4 shows the ratios of Tg bound to LCA related to the total Tg in the serum obtained from each patients. As is clear from Fig. 4, the range of the ratios of normal patient (1) does not overlap with that of benign thyroid adenoma patients (2) or thyroid carcinoma patients (3). It is also found that there is a part of the range of the ratios of thyroid carcinoma patients not overlapped with that of benign thyroid adenoma patients. Namely, it is clear that differentiation diagnosis of thyroid carcinoma or benign thyroid adenoma from normal or that of thyroid carcinoma from benign thyroid adenoma by using the ratios is possible.

[2] The correlation of "the ratio of Tg bound to LCA /total Tg" with an amount of "total Tg." The correlations obtained from normal patients, benign thyroid adenoma patients, or thyroid carcinoma patients are shown in Fig. 5 to 7, respectively.

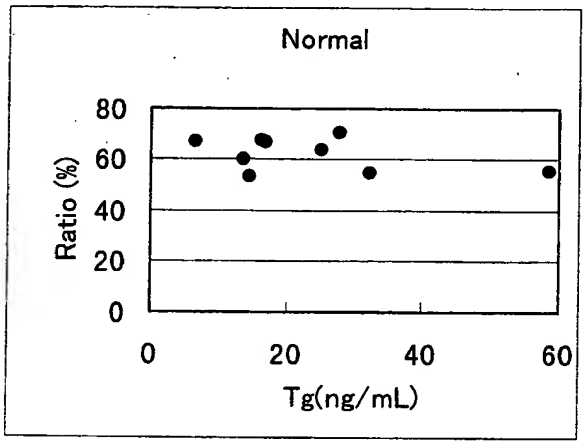


Fig. 5

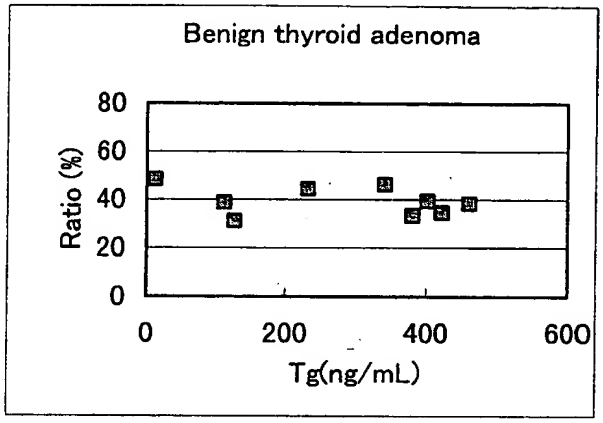


Fig. 6

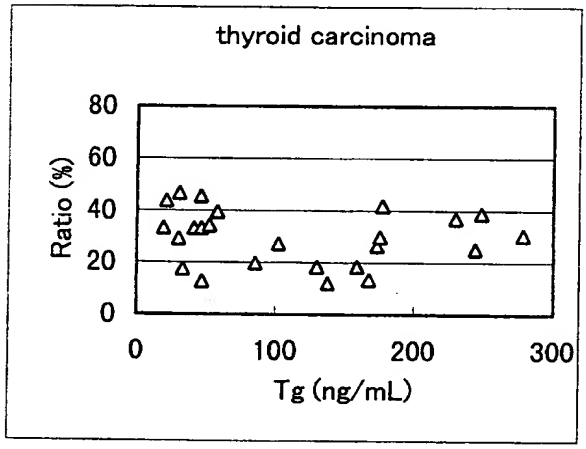


Fig. 7

As is clear from Fig. 5 to 7, the ratio of Tg bound to LCA / total Tg is not correlate with an amount of total Tg in any case of normal patient, benign adenoma patient, or thyroid carcinoma patient. Therefore, it is not taken to mean a greater ratio relative to the total. (That is, it cannot be said that the increase of an amount of total Tg shows the increase of a ratio of Tg bound to LCA / total Tg.)

5. Conclusion

As is clear from the result mentioned above, the use of the amount of total Tg, the amount of Tg bound to LCA or the amount of Tg not bound to LCA is not suitable for a parameter for determination of malignancy of thyroid tumor, since it is impossible to distinguish thyroid carcinoma or benign thyroid adenoma with normal or thyroid carcinoma with benign thyroid adenoma, if these amounts are used as the parameter.

In contrast, when the ratio of Tg bound to LCA/total Tg is used as a parameter for determination of malignancy of thyroid tumor, the correct determination can be performed. That is, the use of the ratio can distinguish thyroid carcinoma or benign thyroid adenoma with normal or thyroid carcinoma with benign thyroid adenoma.

That is, the result mentioned above shows that the use of a ratio as a parameter can distinguish between benign thyroid adenoma and malignant thyroid carcinoma.

On the other hand, none of references including Yamamoto (Eur. J. Biochem., 143, 133-144 (1984)) disclose or suggest a method for distinguishing between benign thyroid and malignant thyroid. That is, Yamamoto only suggests that Tg from malignant thyroid gland is richer in triantennary complex-type oligosaccharides than Tg from normal tissues, though Yamamoto does not disclose or discuss Tg from benign thyroid.

Therefore, the use of a ratio produces unexpected result over the references including Yamamoto.

In conclusion, one of ordinary skill in the art can not have been motivated to use the ratio for determination of malignancy from any references, and moreover, even skilled artisan can not have had a reasonable expectation of success in making a method relied on the calculation and comparison of ratios of a first or second type of Tg (Tg bound to LCA or Tg not bound to Tg) to total Tg.

I, the undersigned declarant, declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and; further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 17 th day of February, 2003.

Kenji Nakamura

Kenji Nakamura